Structural variation and dynamics of the nuclear ribosomal intergenic spacer region in key members of the *Gibberella fujikuroi* species complex

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Abstract: The intergenic spacer (IGS) region of the ribosomal DNA was cloned and sequenced in eight species within the *Gibberella fujikuroi* species complex with anamorphs in the genus *Fusarium*, a group that includes the most relevant toxigenic species. DNA sequence analyses revealed two categories of repeated elements: long repeats and short repeats of 125 and 8 bp, respectively. Long repeats were present in two copies and were conserved in all the species analyzed, whereas different numbers of short repeat elements were observed, leading to species-specific IGS sequences with different length. In *Fusarium subglutinans* and *Fusarium nygamai*, these differences seemed to be the result of duplication and deletion events. Here, we propose a model based on unequal crossing over that can explain these processes. The partial IGS sequence of 22 *Fusarium proliferatum* isolates was also obtained to study variation at the intraspecific level. The results revealed no differences in terms of number or pattern of repeated elements and detected frequent gene conversion events. These results suggest that the homogenization observed at the intraspecific level might not be achieved primarily by unequal crossing-over events but rather by processes associated with recombination such as gene conversion events.

Résumé : L'espaceur intergénique (IGS) au sein de l'ADN ribosomique a été cloné et séquencé chez huit espèces appartenant au complexe d'espèces *Gibberella fujikuroi* ayant des anamorphes au sein du genre *Fusarium*, incluant les plus importantes en matière de production de toxines. L'analyse des séquences d'ADN a révélé deux catégories d'éléments répétés : les répétitions longues et courtes mesurant respectivement 125 pb et 8 pb. Les répétitions longues étaient présentes en deux copies et conservées chez toutes les espèces analysées tandis que le nombre d'éléments répétitions courtes était variable et permettait de distinguer les séquences provenant des diverses espèces sur la base de différences de taille. Chez *Fusarium subglutinans* et *Fusarium nygamai*, ces différences semblaient découler d'évènements de duplication et de délétion. Les auteurs proposent ici un modèle pour expliquer ces processus sur la base de recombinaisons inégales. La séquence IGS partielle chez 22 isolats de *Fusarium proliferatum* a également été examinée afin d'étudier la variation au niveau intraspécifique. Ces analyses n'ont montré aucune différence en termes de longueur ou de type des éléments répétés et ont permis de détecter de nombreux évènements de conversion génique. Ces résultats suggèrent que l'homogénéisation observée au niveau intraspécifique pourrait ne pas être obtenue par le biais de recombinaison inégale, mais plutôt par d'autres processus associés à la recombinaison comme la conversion génique. [Traduit par la Rédaction]

Introduction

The *Gibberella fujikuroi* species complex includes more than nine closely related teleomorph species (sexual states) with anamorph species (asexual states) in the genus *Fusarium* (O'Donnell et al. 1998). From these, *Fusarium proliferatum* (teleomorph *Gibberella intermedia*) and *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) are considered important pathogens of widely distributed wild and cultivated plant species. They also produce a range of secondary metabolites, including harmful mycotoxins such as fumonisins. Because of the relevant implications of these species in the agro-food industry, much effort has been made during the past two decades to detect variability in and design of DNA-based tools to diagnose toxigenic strains as well as for use in phylogenetic analyses. For these purposes, genetic markers based on protein coding and noncoding DNA have been used, among which elongation factor 1-α gene (*EF*-1α) and the nuclear ribosomal DNA intergenic spacer (IGS), respectively, seem to have the most phylogenetic signal (O'Donnell et al. 2009). In filamentous fungi, including *Fusarium* species, rDNA units are present in many copies per genome and are organized as tandem arrays, including highly conserved genes and more variable noncoding spacer regions. These spacer regions are the internal transcribed sequence (ITS) — located between the 18S and 5.8S genes (ITS1) and between the 5.8S and 28S genes (ITS2) — and the IGS region, which separates rDNA repeat units. Owing to the apparent lack of selective constraints, the IGS region is considered to be the most rapidly evolving spacer region within the rDNA array and displays the highest variability (Hillis and Dixon 1991) and thus has been suitable for both phylogenetic analyses (Mirete et al. 2004; Schweigkofler et al. 2004) and diagnostic purposes (Bluhm et al. 2002; Mirete et al. 2003; González-

Table 1. *Fusarium* isolates analyzed in this study indicating host, origin, intergenic spacer (IGS) type, and GenBank accession No. of the corresponding IGS nucleotide sequence.

Isolates[a]	Host	Origin	IGS type[b]	GenBank accession No.[b]
***Gibberella fujikuroi* complex**				
F. verticillioides A0999	*Zea mays*	USA	—	AJ575185
F. sacchari B3852	Laboratory cross		—	AJ879944
F. fujikuroi C1993	*Oryza sativa*	Taiwan	—	AJ879945
F. proliferatum D4854	Laboratory cross		—	AJ879946
F. subglutinans E0990	*Zea mays*	USA	—	AJ879947
F. thapsinum F4094	Laboratory cross		—	AJ879948
F. nygamai G05112	Laboratory cross		—	AJ879949
F. circinatum H69720	*Pinus patula*	South Africa	—	AJ879950
Fusarium proliferatum				
FpB12	*Hordeum vulgare*	Burgos (Spain)	1	GQ495212
FpB23	*Hordeum vulgare*	Burgos (Spain)	1	GQ495217
FpB22	*Hordeum vulgare*	Burgos (Spain)	2	GQ495216
FpB20	*Hordeum vulgare*	Burgos (Spain)	2	GQ495214
FpB21	*Hordeum vulgare*	Burgos (Spain)	2	GQ495215
FpB15	*Hordeum vulgare*	Burgos (Spain)	1	GQ495213
FpO24	*Hordeum vulgare*	Seville (Spain)	1	GQ495220
FpC24	*Hordeum vulgare*	Seville (Spain)	1	GQ495219
FpC3	*Hordeum vulgare*	Seville (Spain)	1	GQ495218
Gf37	*Musa sapientum*	Ecuador	1	GQ495205
Gf33	*Pinus*	Madrid (Spain)	2	GQ495203
Gf31	*Pinus*	Madrid (Spain)	2	GQ495202
Gf29	*Pinus*	Madrid (Spain)	2	GQ495201
Gf34	*Pinus*	Madrid (Spain)	1	GQ495204
Gf26	*Pinus*	Madrid (Spain)	1	GQ495200
FpMM11	*Zea mays*	Madrid (Spain)	1	GQ495193
FpMM12	*Zea mays*	Madrid (Spain)	1	GQ495211
FpMM13	*Zea mays*	Madrid (Spain)	1	GQ495194
FpMM31	*Zea mays*	Madrid (Spain)	1	GQ495195
FpMM41	*Zea mays*	Madrid (Spain)	1	GQ495196
FpMM42	*Zea mays*	Madrid (Spain)	1	GQ495197
FpMM61	*Zea mays*	Madrid (Spain)	2	GQ495198

[a]Isolates from the *G. fujikuroi* species complex A–H (Covert et al. 1999; Kerényi et al. 1999).
[b]IGS types and sequences of *F. proliferatum* were derived from Jurado et al. (2012).

Jaén et al. 2004; Patiño et al. 2004; Sampietro et al. 2010) of closely related *Fusarium* species.

An interesting general feature observed within the rDNA IGS of eukaryotes is the presence of repeated elements. These repeats were previously reported within the rDNA IGS of *Fusarium oxysporum* (Mbofung et al. 2007; O'Donnell et al. 2009) and are well known in other fungi (Ganley and Scott 1998; Guidot et al. 1999; James et al. 2001; Ciarmela et al. 2002; Pantou et al. 2003; Wang et al. 2012). The variation in length among individuals or populations was attributed to variation in the copy number of one or several repeated motifs generated by duplications or deletions during crossing-over events (Coen et al. 1982; Pukkila and Skrzynia 1993). These repeated sequences can operate as *cis*-acting sequences, because this region contains several noncoding functional elements involved in the origin of replication activity and also in ribosomal gene expression such as promoters and enhancers (Ganley et al. 2005). On the other hand, this spacer can also promote unequal crossing over and maintain homogeneity between rDNA repeats of the same organism (Szostak and Wu 1980; Dover 1982). Thus, individual repeats of the whole rDNA unit, including ITS and IGS spacers, evolve in a concerted fashion rather than independently (Nei and Rooney 2005). This is believed to be the result of a phenomenon referred to as concerted evolution, a process that homogenizes all the repeats within the array and fixes the rDNA repeat units within a sexually reproducing species (Dover 1982). Recently, analysis within the rDNA arrays of five fungal species revealed low levels of intragenomic variation, suggesting that concerted evolution is very rapid and efficient (Ganley and Kobayashi 2007). The three possible recombination mechanisms that can lead to changes in the number of rDNA repeat units on a chromosome are sister chromatid exchange and interchromosomal and intrachromosomal exchange (Eickbush and Eickbush 2007). Experiments carried out in yeast suggest that the major mechanism accounting for concerted evolution is unequal crossing over as a result of sister chromatid exchange (Petes 1980; Szostak and Wu 1980), but gene conversion can also play an important role in the homogenization of rDNA units (Eickbush and Eickbush 2007). Gene conversion events can occur between homologous, as well as nonhomologous, chromosomes and thus can readily lead to the homogenization of rDNA units in a population (Dover 1982; Eickbush and Eickbush 2007). Nevertheless, the relative roles in homogenization carried out by unequal crossing over and gene conversion have yet to be elucidated.

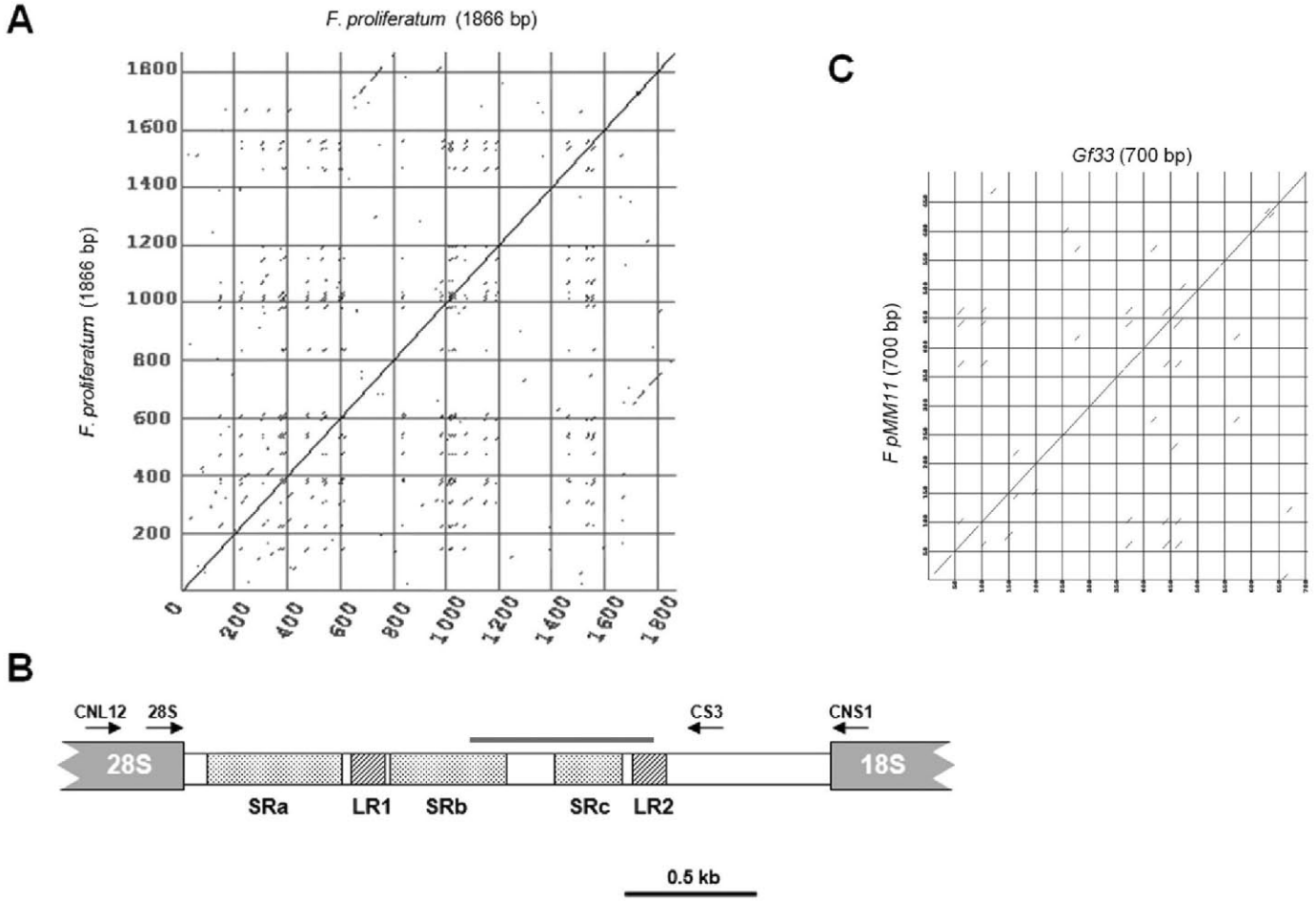
In this study we aimed to characterize the primary structure and dynamics of the nuclear ribosomal IGS region in closely related species of the *G. fujikuroi* species complex. To this end, we compared the aligned IGS sequences of these species and searched for repeated elements using dot plot analyses. In addition, recombination events that could eventually be caused by gene conversion mechanisms were analyzed both at the interspecific level in key members of the *G. fujikuroi* species complex as well as at the intraspecific level in the fumonisin-producing *F. proliferatum*, one of the most diverse species of this complex in terms of host and geographic distribution.

Material and methods

Fungal isolates and growth conditions

The strains of *Fusarium* used in this study are listed in Table 1. Single-spore cultures were maintained on potato dextrose agar

Fig. 1. Structure and organization of the intergenic spacer (IGS) region within the *Gibberella fujikuroi* species complex. (A) Dot plot self-comparison of the IGS sequence of *Fusarium proliferatum*. Diagonal bars represent direct repeated elements. The numbers 1866 and 700 indicate the length of the sequence. (B) Schematic diagram of the IGS region of the *G. fujikuroi* species complex. This sequence contains two long repeats (LR1 and LR2) and several short repeat (SR) elements distributed upstream of the LR1, denoted SRa, and upstream of the LR2, denoted SRb and SRc. PCR primers 28S and CS3, as well as primers CNL12 and CNS1, are indicated by arrows. The grey bar above the IGS region indicates the partial IGS sequence analyzed in 22 isolates of *F. proliferatum*. (C) Dot plot comparison between the IGS sequences of FpMM11 and Gf33 isolates of *F. proliferatum*.



(Scharlau Chemie, Barcelona, Spain) at 4 °C and stored as spore suspension in 15% glycerol at −80 °C. The isolates were cultured in 100 mL Erlenmeyer flasks containing 20 mL Sabouraud liquid medium (Scharlau Chemie). Cultures were inoculated with mycelial disks cut from the margins of 7-day-old colonies and incubated at 25 °C under static conditions. Mycelia from 2-day-old cultures were harvested by filtration through Whatman No. 1 paper (Whatman International Ltd., Maldstone, UK), immediately frozen in liquid nitrogen, and kept at −80 °C for DNA isolation.

DNA extraction, PCR amplification, cloning, and sequencing

Fungal mycelium was frozen with liquid nitrogen and ground to a fine powder in a mortar. Subsequently, total genomic DNA extraction was performed using a genomic DNA extraction kit (Genomix, Talent, Trieste, Italy).

The IGS region of the eight species of the *G. fujikuroi* complex was amplified with primers 28S (5′-GTCCTGTAAGCAGTAGAG-3′) and CS3 (5′-GCCGGATTTGCTCCCTTCTC-3′). These primers can specifically amplify the IGS region where repeated elements are present. Primer 28S was located 103 bp downstream of the CNL12 primer, and primer CS3 was located 573 bp upstream of the CNS1 primer (Fig. 1B). Primers CNL12 and CNS1 were described previously (Appel and Gordon 1995). PCR was performed in an Eppendorf Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) using the following program: 1 cycle of 85 s at 94 °C, 35 cycles of 35 s at 95 °C (denaturalization), 55 s at 58 °C (annealing), 2 min at 72 °C (extension), and finally 1 cycle of 10 min at 72 °C. Each reaction mixture contained 50 ng of template DNA, 1.25 μL of each primer (20 μmol/L), 0.2 μL of *Taq* DNA polymerase (5 U/μL), 2.5 μL of 10× PCR buffer, 1 μL of $MgCl_2$ (50 mmol/L), and 0.25 μL of dNTPs (100 mmol/L) supplied by the manufacturer (Ecogen, Barcelona, Spain), up to a total volume of 25 μL. Amplification products were analyzed by electrophoresis in agarose gels. A single band with an approximate size of 1.8 kb was obtained for each species, excised from the gel, purified, and cloned with the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif., USA). One clone was sequenced on both strands for each species using M13 reverse and T7 forward primers. IGS sequences were accomplished by primer walking. Sequences were obtained using an ABI PRISM DNA sequencer (Applied Biosystems, Foster City, Calif., USA) at the Genomic Unit of the Complutense University of Madrid (Spain) and CIB-CSIC (Madrid, Spain).

Sequence and phylogenetic analyses

Dot plot analyses, alignments, and nucleotide similarities of the IGS region sequences were conducted with the computer program MegAlign of the Dnastar package (Lasergene, Madison, Wis., USA).

Table 2. Nucleotide similarity (%), length, and number of short repeat (SR) elements within the IGS region of the *Gibberella fujikuroi* species complex.

	Fve	Fsa	Ffu	Fpr	Fsu	Fth	Fny	Fci
Fve	100	85.4	86.0	85.0	86.9	89.7	92.7	87.3
Fsa		100	89.8	88.2	85.7	83.3	86.8	85.9
Ffu			100	92.4	85.8	84.3	87.9	86.3
Fpr				100	84.7	83.4	86.6	85.1
Fsu					100	85.0	88.0	97.1
Fth						100	92.5	85.7
Fny							100	88.7
Fci								100
Length (bp)	1870	1864	1864	1866	1936	1883	1732	1856
No. of SRs	20	20	20	20	24	19	16	19

Note: Species abbreviations are as follows: Fve, *Fusarium verticillioides*; Fsa, *F. sacchari*; Ffu, *F. fujikuroi*; Fpr, *F. proliferatum*; Fsu, *F. subglutinans*; Fth, *F. thapsinum*; Fny, *F. nygamai*; Fci, *F. circinatum*.

To detect repeated elements within the IGS region, dot plot analyses were performed. Dot plot is a graphical method that compares two sequences by overlapping them, allowing the detection of matching regions with a user-specified number of residues (window size) and with a user-specified similarity (percent match). These matching regions are then displayed as a line on the dot plot. In this study, dot plot analyses were performed with a window size of 10 and a percent match of 85% using the MegAlign program. Alignments of entire and partial IGS region sequences of eight species of the *G. fujikuroi* species complex and 22 *F. proliferatum* isolates, respectively, were carried out using the ClustalW program as implemented in MegAlign. Nucleotide similarities of the IGS region sequences of eight species of the *G. fujikuroi* species complex were determined based on a ClustalW alignment. To provide statistical evidence for recombination, sequences were analyzed with the computer program GENECONV v.1.81 (Sawyer 1999).

Phylogenetic analyses were performed using PAUP 4.0 b10 (Swofford 2002). Gaps were coded as missing data and were excluded from the analyses. Unweighted parsimony analyses were performed on the data set using the heuristic search option with 1000 random addition sequences and tree bisection–reconnection branch swapping. Clade stability was assessed by 1000 bootstrap replications (Hillis and Bull 1993).

Results

IGS sequence analysis

We analyzed cloned fragments, including most of the IGS region of the eight species of the *G. fujikuroi* species complex and previously reported (Jurado et al. 2012) partial 700-bp IGS sequences of 22 *F. proliferatum*. Sequence analysis revealed that IGS sequences varied in size from 1732 bp in *F. nygamai* to 1936 bp in *F. subglutinans* (Table 2). At the 3′ end of the IGS-sequenced region, a putative transcription initiation site was identified (TATATGAGAA). A putative termination transcription site for RNA polymerase I was found in the IGS region of all the *G. fujikuroi* species analyzed and was similar to the termination transcription site reported for *Schizosaccharomyces pombe* (Melekhovets et al. 1997). A sequence similar to the topoisomerase I (TOPO I) recognition sequence, 5′-CTTAGA-3′, similar to the one reported for *Tetrahymena thermophila* (Bonven et al. 1985), was found in the IGS region of *F. verticillioides*, *F. sacchari*, *F. fujikuroi*, *F. subglutinans*, *F. nygamai*, and *F. circinatum*.

Dot plot analysis was used to detect repeated elements in pairwise comparisons (Fig. 1A), a procedure that enabled tracing of its structural organization (Fig. 1B). These repeated elements were classified into two categories based on their number, sequence length, and nucleotide similarity: short repeat (SR) and long repeat (LR). Two LRs of 125 bp, separated from one another by 850 bp and having a nucleotide similarity of 86%, were found in the IGS sequence. A variable number of SRs were found in each species, with a length of 8 bp and a conserved sequence of 5′-AGGGTAGG-3′. These short repeats were present throughout the whole IGS and were localized upstream of the LRs in three areas referred to as SRa, SRb, and SRc.

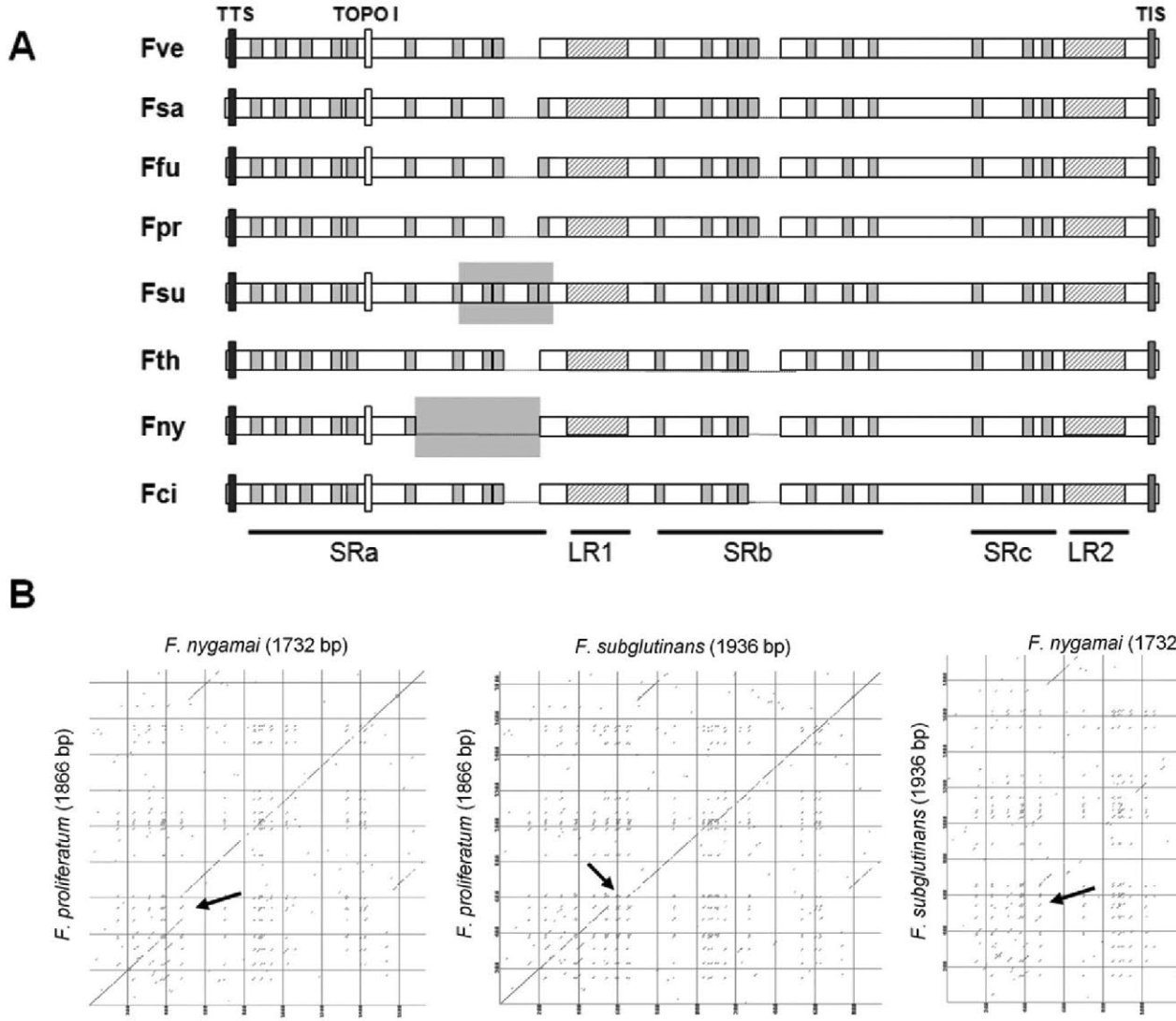
Comparison of IGS sequences

IGS sequences from the eight species showed a DNA sequence similarity that ranged between 61.3% (*F. subglutinans* – *F. proliferatum*) and 95.7% (*F. circinatum* – *F. subglutinans*), as shown in Table 2. Schematic representations of the IGS region revealed the presence of conserved SR and LR elements among the eight IGS sequences compared (Fig. 2A). Two LRs, denoted LR1 and LR2, were observed in all pairwise comparisons. LR1 was located in the initial region of the IGS and was found to be more variable among species than LR2 in terms of nucleotide similarity (85% and 98%, respectively). Another interesting feature of LR elements is that they were also detected in the closely related species *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *radicis lycopersici*, as well as within *F. equiseti* (data not shown).

Although the IGS organization was found to be conserved among the species analyzed, the number and the distribution pattern of SR elements were different in some species (Table 2; Fig. 2A). Thus, the number of SRs varied from 16 to 24 copies in *F. nygamai* and *F. subglutinans*, respectively. Dot plot analyses showed the presence of an insertion and a deletion within the IGS region of *F. subglutinans* and *F. nygamai*, respectively, when they were compared with *F. proliferatum* and between each other (Fig. 2B). Thus, the actual sequence from *F. subglutinans* appeared to be generated by a duplication of a 50-bp segment between a single SR and two adjacent SRs, located at 550 and 600 bp, respectively. In the *F. nygamai* IGS sequence, a deletion of a 200-bp fragment located at 478 bp was observed in the alignment. These results suggest that the current IGS sequences of these species underwent unequal crossing-over events of their ancestral sequences between SRs elements, as described in the model shown in Fig. 3. These processes enabled the generation of the indels found in SR-rich areas that gave rise to the variation in size observed in the sequences of *F. nygamai* and *F. subglutinans*. In addition, an insertion of 15 bp in *F. subglutinans* between two SR sequences was detected, located at 1109 bp. In the IGS of *F. verticillioides* and *F. thapsinum*, an insertion of 8 bp next to an SR element occurred, located at 1247 bp.

Gene conversion processes are considered to play an important role in the homogenization of rDNA units. To detect the presence of gene conversion events, we performed a pairwise test by using the program GENECONV over an alignment of 1988 bp within the eight species of the *G. fujikuroi* species complex. GENECONV performs a pairwise comparison of similar sequences in a given alignment and assign a *P* value expected for that pair of sequences in the absence of gene conversion. As a result, this program detected five tracts linked to gene conversion, as shown in Table 3. Two gene conversion pairs

Fig. 2. Comparison of the intergenic spacer (IGS) sequence organization among eight species of the *Gibberella fujikuroi* species complex. (A) Schematic representation of the IGS region showing both types of repeats, short (SR) and long (LR). The dotted lines indicate an indel. Regions duplicated and deleted are shaded in grey within the IGS of *Fusarium subglutinans* and *F. nygamai*, respectively. In the line below are indicated the regions of LR and SR elements. Putative transcription initiation site (TIS), transcription termination site (TTS), and topoisomerase I (TOPO I) recognition sequence are also shown. Fve, *F. verticillioides*; Fsa, *F. sacchari*; Ffu, *F. fujikuroi*; Fpr, *F. proliferatum*; Fsu, *F. subglutinans*; Fth, *F. thapsinum*; Fny, *F. nygamai*; Fci, *F. circinatum*. (B) Dot plot comparison of the IGS of *F. proliferatum* with *F. subglutinans* and *F. nygamai* and between *F. subglutinans* and *F. nygamai*. Arrows in *F. subglutinans* and *F. nygamai* indicate an indel. The number following each name indicates the length of the sequence.



were detected in both *F. fujikuroi* and *F. nygamai*, whereas *F. proliferatum* did not participate in any gene conversion event. Two gene conversion pairs mapped in the same fragment (from 1496 to 1562 bp): between *F. subglutinans* and *F. nygamai* and between *F. circinatum* and *F. nygamai*. Given the high nucleotide similarity observed between IGS sequences from *F. circinatum* and *F. subglutinans*, this result suggest an ancestral gene conversion event where a fragment from *F. nygamai* was replaced by another fragment from the *F. subglutinans* and *F. circinatum* clade.

Phylogenetic analyses

A phylogenetic tree was constructed using PAUP containing the IGS sequences of the *G. fujikuroi* species complex analyzed in this work (Fig. 4). The phylogeny revealed homoplasy, as indicated by the low consistency index obtained, although the data support the monophyly of two main clusters with high bootstrap values. The first cluster comprised *F. fujikuroi*, *F. proliferatum*, and *F. sacchari* species, all of which shared the same array of SR elements in terms of number and position. The second cluster, consisting of *F. circinatum* and *F. subglutinans*, forms a sister group to *F. thapsinum*, *F. nygamai*, and *F. verticillioides*. In this cluster, IGS sequences contained different numbers of SR elements and evolved faster as reflected by their branch lengths. Thus, in the IGS sequences of *F. thapsinum*, *F. nygamai*, and *F. verticillioides*, the number of SR repeats was 19, 16, and 20, respectively. On the other hand, *F. circinatum* and *F. subglutinans*, which were closely related, contained 19 and 24 repeats, respectively. These results suggest that the sequence of these five species might share an ancestral IGS array that evolved independently in each species, not only by the accumulation of base substitutions but also by the presence of different indels.

Intraspecific comparison of the IGS organization

To assess the extent of IGS variation at the intraspecific level, we examined the partial IGS sequences of 22 *F. proliferatum* isolates

Fig. 3. A model of the sequence dynamics that generated the actual intergenic spacer (IGS) sequences of *Fusarium subglutinans* and *F. nygamai*. The 50-bp insertion in *F. subglutinans* was accomplished by an unequal crossing-over event between two ancestral IGS sequences. The IGS structure of these ancestral IGS sequences is the same as that found within the IGS sequence of *F. circinatum*, a species closest to *F. subglutinans*. A subsequent unequal crossing-over event between the previous shorter product and any other sequence containing up to the sixth white box (including the previous shorter product) might give rise to the observed IGS sequence of *F. nygamai* and to a longer IGS sequence that may not have been fixed. The boxes with diagonal bars indicate a long repeat (LR) element (LR1) and the grey boxes the short repeat (SR) elements within the SRa region.

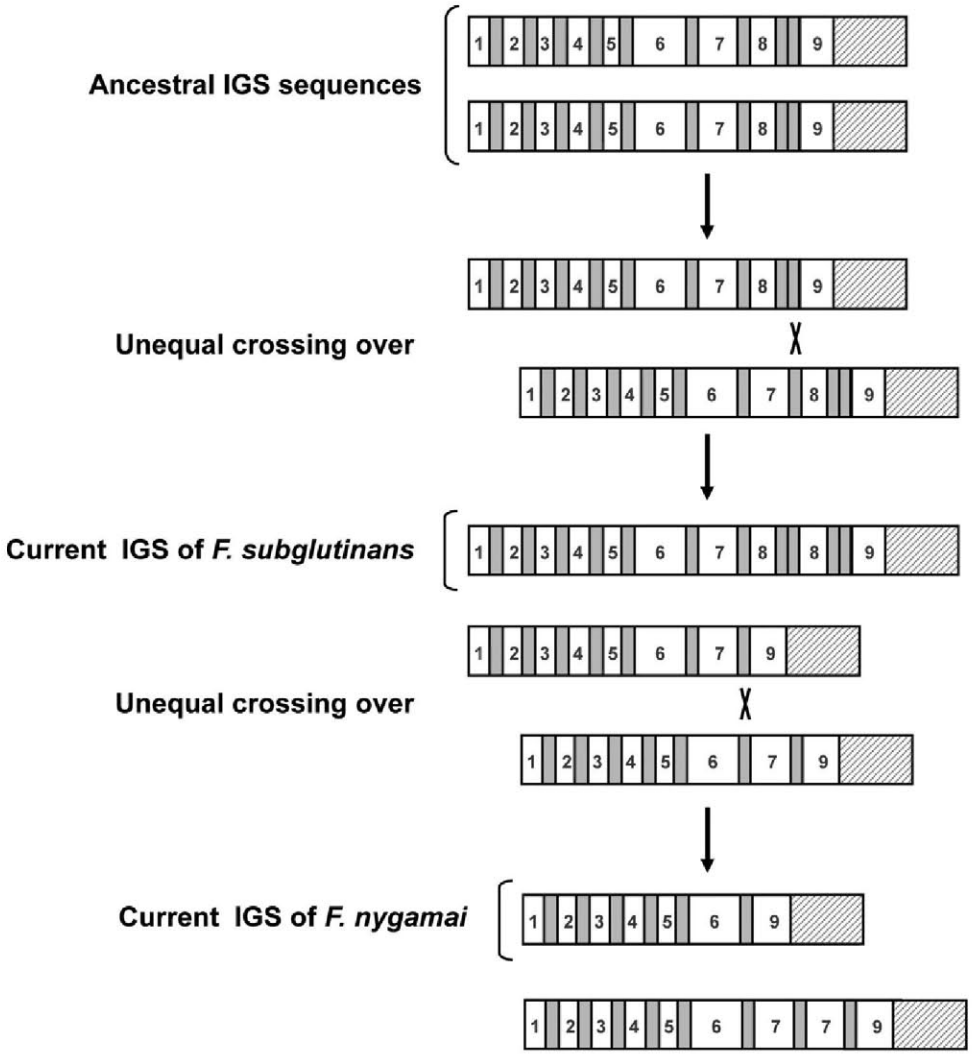


Table 3. Interspecific gene conversion analysis performed with GENECONV based on a ClustalW alignment.

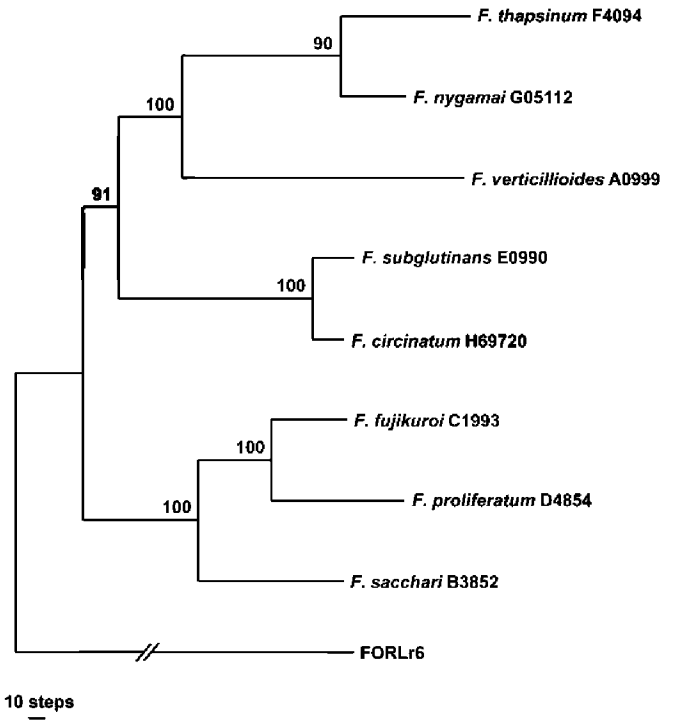
Pairwise comparison		Converted fragment			Simulated *P*
		Begin	End	Length	
F. verticillioides	*F. circinatum*	1133	1181	49	0.0414
F. fujikuroi	*F. sacchari*	616	734	119	0.0059
F. fujikuroi	*F. thapsinum*	1726	1781	56	0.0373
F. nygamai	*F. subglutinans*	1496	1562	67	0.0066
F. nygamai	*F. circinatum*	1496	1561	66	0.0200

that expanded from 1185 to 1908 bp (Fig. 1C), corresponding to the most variable region detected in the IGS sequence (Mirete et al. 2004). These isolates were derived from different hosts and locations and also shared different IGS types (Table 1) as previously reported (Jurado et al. 2012). Dot plot and sequence analyses across these isolates revealed that both the number and distribution of SRs were conserved. A conserved pattern of SR and LR elements was also observed in the entire IGS sequences of three *F. verticilliodes* isolates (GenBank accession Nos. AJ880004, AJ880005, and AJ880006) (data not shown). Within the partial IGS sequences of *F. proliferatum* isolates, a total of 40 gene conversion events were detected using the program GENECONV (Table 4). Four fragments were found to undergo gene conversion and expanded from 364 to 392, from 482 to 540, from 239 to 329, and from 239 to 358. The first two fragments had converted between IGS type 1 and IGS type 2 sequences, whereas the latter two had exchanged only between IGS type 2 sequences.

Discussion

Comparative analysis of the primary structure of the IGS region was considered in this study for deciphering the structure and dynamics of repeats and (or) functional motifs in members of the *Gibberella fujikuroi* species complex. The consensus sequence, as well as the number and distribution of SR and LR repeated elements identified in the present study, was similar to those detected in the central portion of the IGS rDNA of the *F. oxysporum* species complex (Mbofung et al. 2007; O'Donnell et al. 2009). Thus, we identified 8-bp repeated elements with a consensus sequence

Fig. 4. One most parsimonious (MP) tree inferred from the intergenic spacer (IGS) sequence data set of the *Gibberella fujikuroi* isolates with assigned bootstrap values. *Fusarium oxysporum* FORLr6 was used as an outgroup (GenBank accession No. AJ880007). Tree length = 1034 steps; consistency index (CI) = 0.7950; retention index (RI) = 0.6461.



of AGGGTAGG, whereas in *F. oxysporum* a consensus sequence of GGTGTAGGGTAGG was reported for these repeats. In addition, two different repeats of 15 and 20 bp identified in *F. oxysporum* were also present within each LR element identified here. Nevertheless, the differences found in the consensus sequences may be attributed to the different bioinformatic analyses and parameters applied to detect repeated sequences rather than to sequence divergence between these two species complexes. Similar sequences to the 8-bp SR elements have been previously identified within the IGS sequence of the entomopathogenic fungus *Metarhizium* as CGGGTAGG (Pantou et al. 2003) and the genus *Tuber* as AGGGTAAC (Ciarmela et al. 2002), near the end of the 28S and 25S rRNA, respectively. These repeated elements may be involved in the transcriptional termination and (or) maturation of the 35S rRNA, since this region is known to contain several regulatory motifs and signals (Dutta and Verma 1990; Ganley et al. 2005). From these repeats elements, LRs were conserved across species, with the most conserved regions within IGS being those surrounding these repeats. The close similarity between LR1 and LR2 repeats, in combination with their position near the 28S and 18S genes, respectively, may be related to a functional role in transcription or in maturation of the precursor rRNA (Pantou et al. 2003). LRs showed high conservation among the species analyzed and may have arisen by a duplication event. In addition, LR1 is more variable than LR2, suggesting a stronger selection pressure in LR2. These regions are good candidates for additional regulatory motifs.

SR elements showed variability among species in terms of number and position. Indels were restricted to SR-rich areas, suggesting that these elements are involved in the difference in length observed. In addition, we found evidence that duplication and deletion events in the IGS region of *F. subglutinans* and *F. nygamai* gave rise to the differences in length and also to the different number of repeats. These mutations can be produced by unequal crossing over, which has been suggested as one of the major driving forces in the evolution of the rDNA units (Eickbush and Eickbush 2007). In species with asexual reproduction, mitotic crossing over can have an important impact on concerted evolution and can be produced between sister chromatids or between repeats (intrachromatid recombination) (Ganley and Scott 1998; James et al. 2001). These latter two processes are considered by some authors to be the most important contribution to concerted evolution, probably occurring at a higher rate than conventional (meiotic) events (Schlötterer and Tautz 1994). The simple sequence motifs within the repeat arrays, in addition to the LR elements, may act as recognition sites for the initiation of recombination. The presence of at least one TOPO I site may facilitate chromosomal and (or) chromatid exchange. The TOPO I site is believed to play a role in recombination events within the IGS region of *Drosophila* (Polanco et al. 1998) and has been found as well within the IGS1 region of *Saccharomyces* (Ganley et al. 2005). In human rDNA, recombination through TOPO I may homogenize specific regions between TOPO I sites (Gonzalez and Sylvester 2001). Thus, we hypothesized that these duplication and deletion events may have occurred via unequal crossing over via mitotic recombination.

On the other hand, the phylogenetic tree shown in Fig. 4 revealed that *F. thapsinum* contains an IGS region sequence that is

Table 4. Intraspecific gene conversion analysis performed with GENECONV based on a ClustalW alignment.

Pairwise comparison		Converted fragment			Simulated *P*
		Begin	End	Length	
FpMM11	FpMM61	364	392	29	0.0495
FpMM11	FpB20	364	392	29	0.0495
FpMM12	FpMM61	364	392	29	0.0495
FpMM12	FpB20	364	392	29	0.0495
FpMM13	FpMM61	364	392	29	0.0495
FpMM13	FpB20	364	392	29	0.0495
FpMM31	FpMM61	364	392	29	0.0495
FpMM31	FpB20	364	392	29	0.0495
FpMM42	FpMM61	364	392	29	0.0495
FpMM42	FpB20	364	392	29	0.0495
FpMM61	Gf37	364	392	29	0.0495
FpMM61	Gf26	364	392	29	0.0495
FpMM61	Gf34	364	392	29	0.0495
FpMM61	FpO24	364	392	29	0.0109
FpMM61	FpC3	364	392	29	0.0495
FpMM61	Fpc24	364	392	29	0.0109
FpB20	Gf37	364	392	29	0.0495
FpB20	Gf26	364	392	29	0.0495
FpB20	Gf34	364	392	29	0.0495
FpB20	FpO24	364	392	29	0.0109
FpB20	FpC3	364	392	29	0.0495
FpB20	Fpc24	364	392	29	0.0109
FpB22	FpO24	364	392	29	0.0220
FpB22	Fpc24	364	392	29	0.0220
FpO24	FpB21	364	392	29	0.0220
FpB21	Fpc24	364	392	29	0.0220
Gf29	FpO24	482	540	59	0.0474
Gf29	Fpc24	482	540	59	0.0474
Gf31	FpO24	482	540	59	0.0370
Gf31	Fpc24	482	540	59	0.0370
Gf33	FpO24	482	540	59	0.0381
Gf33	Fpc24	482	540	59	0.0381
FpMM61	Gf29	239	329	91	0.0294
Gf29	FpB20	239	329	91	0.0294
Gf29	FpB22	239	329	91	0.0293
Gf29	FpB21	239	329	91	0.0293
FpMM61	Gf31	239	358	120	0.0035
Gf31	FpB20	239	358	120	0.0035
Gf31	FpB22	239	358	120	0.0032
Gf31	FpB21	239	358	120	0.0032

more similar to that of *F. nygamai*, but differed in its IGS structure, as indicated in the alignment shown in Fig. 2A. Therefore, these data suggest that completely independent recombination events may lead to the current IGS structure of *F. subglutinans* (American origin) and *F. nygamai* (African origin). These processes may have accompanied the radiation of these species according to their different biogeographic origin (O'Donnell et al. 1998).

The mechanisms leading to homologous and nonhomologous chromosome exchanges are not clear, and gene conversion may play an important role (Dover 1982), although direct evidence for its involvement is difficult to obtain (Eickbush and Eickbush 2007). The subsequent fixation of a particular IGS sequence within a population may be dependent on meiotic recombination (Appel and Gordon 1996). Therefore, it would be expected that reproductively isolated species would have different IGS patterns. In this study, all the species analyzed within the *G. fujikuroi* species complex could be considered biological (Leslie 1995), morphological (Nirenberg and O'Donnell 1998), and phylogenetic (O'Donnell et al. 1998) species. Also, these species are considered to be reproductively isolated for a long time (Leslie 1995; O'Donnell et al. 1998). Nevertheless, the gene conversion event detected between *F. nygamai*, *F. subglutinans*, and *F. circinatum* suggests that these species may have undergone recombination to some extent during their evolutionary history. In addition, the homoplasy detected in the phylogenetic analyses reflects the likely occurrence of recombination among IGS sequences and would explain the discrepancies observed between the phylogeny obtained in this work when compared with the one reported by O'Donnell et al. (1998) using other genetic markers. On the other hand, our analyses at the intraspecific level within nonorthologous IGS types indicate that recurrent gene conversion events may be taking place and thus contributing to the homogenization of these sequences, including both IGS types. These gene conversion mechanisms may be associated with meiotic recombination, not only because sexual reproduction is relatively common in *F. proliferatum* (Leslie and Klein 1996), but also because *MAT-1* and *MAT-2* alleles are represented in both IGS types within these populations (Jurado et al. 2012). Despite the high intraspecific variability previously reported in these isolates (Jurado et al. 2010, 2012), we concluded that their IGS sequences, as well as those retrieved from *F. verticillioides* isolates (data not shown), evolved in a concerted fashion, since they shared the same number and distribution of SR elements. In addition, the intraspecific IGS variation observed among isolates of *F. oxysporum* with a predominantly clonal mode of reproduction (Appel and Gordon 1996) supports the notion that the homogenization of the IGS sequence would occur at a higher rate in species with sexual reproduction such as *F. proliferatum* and *F. verticilliodes*.

The presence of high levels of nucleotide polymorphism within the IGS rDNA provides a high degree of phylogenetic resolution that enables the discrimination of *Fusarium* individuals or populations with different toxigenic profiles or ecophysiological characteristics (Mirete et al. 2003, 2004; González-Jaén et al. 2004; Hinojo et al. 2004; Patiño et al. 2004, 2006; Jurado et al. 2006; Sampietro et al. 2010; Marín et al. 2012). Nevertheless, previous studies have shown discordance between the IGS rDNA and the *EF-1α* gene within members of the *G. fujikuroi* species complex (Mirete et al. 2004) and within the *F. oxysporum* species complex (Mbofung et al. 2007; O'Donnell et al. 2009). In *F. oxysporum*, the incongruence was attributed to the nucleotide variability localized outside the repeated elements rather than sequence variation within or different numbers of these elements (O'Donnell et al. 2009). Given the high conservation of the repeats found within the IGS sequences analyzed, our results suggest that conflicting relationships between the IGS rDNA and other loci such as the *EF-1α* gene and *MAT* alleles may be due to the underlying genetic mechanisms described in the present study.

In summary, our results suggest that the two major mechanisms involved in the sequence homogenization of rDNA units, unequal crossing over and gene conversion processes, may operate to some extent within the IGS region of members of this group of *Fusarium* species. Although we could not assess the relative contribution of these processes to the sequence homogeneity observed, our results suggest that gene conversion may play an important role, particularly at the intraspecific level. Overall, these data point out that the IGS rDNA region can be a useful additional marker of evolutionary dynamics of divergent species or populations, to detect genetic variability, to develop diagnostic markers, and for phylogenetic analyses, when enough information is available on the IGS structure and the extent of sexual reproduction.

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